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## Degradation of 2-Chloroethylvinylether by *Ancylobacter aquaticus* AD25 and AD27

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Incubation of five different  $\beta$ -chloroethers with slurries prepared from brackish water sediment or activated sludge revealed that bis(2-chloroethyl)ether and 2-chloroethylvinylether (2-CVE) were biodegradable under aerobic conditions. After enrichment, two different cultures of *Ancylobacter aquaticus* that are capable of growth on 2-CVE were isolated. Both cultures were also able to grow on 1,2-dichloroethane. The cells contained a haloalkane dehalogenase that dehalogenated 2-CVE, 2-chloroethylmethylether, 2-bromoethylether, and epichlorohydrin. Experiments with cell extracts indicated that an alcohol dehydrogenase and an aldehyde dehydrogenase were also involved in the degradation of 2-CVE. This suggests that 2-CVE is metabolized via 2-hydroxyethylvinylether and vinyloxyacetaldehyde to vinyloxyacetic acid. Enzymatic ether cleavage was not detected. 2-CVE was also degraded by chemical ether cleavage, leading to the formation of 2-chloroethanol and acetaldehyde, both of which supported growth. We propose that *A. aquaticus* strains may be important for the detoxification and degradation of halogenated aliphatic compounds in the environment.

Chlorinated ethers are synthesized industrially in large amounts for various purposes, such as production of anesthetics, sedatives, and cellulose ethers (3). They are also formed as by-products of chemical reactions (for example, during the addition of chlorine to alkenes in a aqueous medium) (4). The unwanted synthesis of several types of different chloroethers during the manufacture of epichlorohydrin has also been described (4).

Chloroethers have been detected in industrial effluents, in air samples, and in river sediments (3, 20). It has been proposed that the predominant process for removal of 2-chloroethylvinylether (2-CVE) from the environment is volatilization into the atmosphere (3). It is thought that this compound undergoes rapid destruction by photooxidation. The mechanism of decomposition involves electrophilic attack by hydroxyl radicals, ozone, or other oxidants on the double bond (3).

Little is known about the bacterial degradation of halogenated ethers. Previously, we described growth of *Pseudomonas* sp. strain AD1 and *Arthrobacter* sp. strain AD2 on epichlorohydrin (3-chloro-1,2-epoxypropane) (22). Epichlorohydrin can be converted to glycerol by an epoxide hydrolase (7, 22) and a haloalcohol dehalogenase (23). 2-CVE and bis(2-chloroethyl)ether can also be dehalogenated hydrolytically by the haloalkane dehalogenase of the gram-positive strain GJ70 (8), producing the corresponding alcohols. To our knowledge, bacterial growth on such chloroethers has not been reported previously. Hydroxyethers like ethoxyethanol, diethylene glycol, and polyethylene glycols are good growth substrates for a variety of microorganisms (2, 6, 14, 15, 17), and much is known about the regulation and biochemistry of the enzymes involved in the degradation of these compounds (2, 14). The information described above indicates that the enzymes catalyzing the dehalogenation and further degradation of 2-CVE and bis(2-chloroethyl)

ether may be present in nature, suggesting that bacterial growth on these compounds is possible.

In this report, we present data on the biodegradation of several chloroethers and the growth of two different *Ancylobacter aquaticus* strains on 2-CVE. The strains were also able to grow on 1,2-dichloroethane. We found that the organisms synthesize enzymes for the degradation of 2-CVE and also can grow on its spontaneously formed degradation products.

### MATERIALS AND METHODS

**Degradation experiments and enrichments.** Degradation experiments were performed in 500-ml serum flasks which contained 120 ml of mineral salts medium supplemented with 30 mg of yeast extract (Difco) per liter (24) and 30 ml of brackish water sediment (90 to 110 mg [dry weight] per ml) or 30 ml of activated sludge (10 to 15 mg [dry weight] per ml). After addition of a haloether at a concentration of 0.5 mM (calculated as if the compound was completely dissolved in the suspension), the flasks were incubated by rotating them at 25 rpm at room temperature. Samples of the slurries were obtained as described previously (18). The isolation of *A. aquaticus* AD25 and AD27 from these enrichment cultures has been described elsewhere (24).

**Organisms and growth conditions.** The pure cultures used in this study were *A. aquaticus* AD25 and AD27, which were characterized and identified as described previously (24). Strain AD25M3 is an alcohol dehydrogenase-negative mutant of strain AD25 and was selected on the basis of resistance to 2-bromoethanol. The properties of *Xanthobacter autotrophicus* GJ10 have also been described previously (12). All strains were routinely grown in mineral salts medium supplemented with 30 mg of yeast extract per liter (pH 7) at 30°C with rotary shaking (24).

Bacterial growth was followed turbidimetrically at 450 nm ( $A_{450}$ ). The strains were pregrown on methanol, and each flask was inoculated with 2% (vol/vol) of a preculture grown on the same medium. In the case of strain AD25, which grew

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in flocks, a reproducible growth rate measurement was possible after the destruction of the bacterial flocks. This was accomplished by pulling 8 ml of culture fluid 20 times through a 10-ml syringe with a needle that had a diameter of 1 mm.

Chloride levels in the cultures were measured by the colorimetric method used previously (24).

**Preparation of crude extracts and enzyme assays.** Enzyme assays were performed with extracts prepared (24) from cultures grown on 2-CVE or from cultures grown on 2 mM glucose which were incubated with 1.5 mM 2-CVE for 24 h prior to harvest.

The haloalkane dehalogenase activities (24), phenazine methosulfate (PMS)-linked alcohol dehydrogenase activities (10), and NAD-dependent aldehyde dehydrogenase activities were measured as described previously (10). The  $V_{\max}$  and  $K_m$  values of the dehalogenase were determined by measuring the initial rates of halide release at various substrate concentrations, followed by nonlinear regression fitting to the Michaelis-Menten equation.

Oxygenase activities were assayed at 30°C with an oxygen electrode. The incubation mixtures contained in a final volume of 4 ml 100 mM potassium phosphate buffer (pH 8), 62.5 mM diglycolic acid or ethoxyacetic acid (both neutralized with 1 N sodium hydroxide), and 1 mM NADH. The reaction was started by adding a suitable amount of crude extract. Diglycolic acid oxidase and diglycolic dehydrogenase activities were assayed by the methods described by Kawai (13).

Enzyme activities and oxygen consumption rates are expressed in units. One unit is defined as the activity that catalyzed the formation of 1  $\mu$ mol of product (halide or NADH) per min or the consumption of 1  $\mu$ mol of oxygen per min. Protein concentrations were determined with Coomassie brilliant blue.

**Experiments with washed cells.** Cells from late-exponential-phase cultures were harvested by centrifugation at  $10,000 \times g$  for 10 min, washed in 50 mM potassium phosphate buffer (pH 7.5), and suspended in an appropriate amount of this buffer.

For the synthesis of vinylxyacetic acid (VAA) by strain AD25, 400-ml cultures were grown on 2.5 mM glucose. After centrifugation at  $10,000 \times g$  for 10 min, the cells were resuspended in growth medium containing no yeast extract, washed once with centrifugation at  $10,000 \times g$  for 10 min, and resuspended in 600 ml of the same medium. The resulting cell suspension was incubated in a closed 1-liter fermentor. The stirring rate was 400 rpm, and the temperature was kept at 30°C by an infrared lamp connected to a temperature sensor and controller. The pH was maintained at 7.1 by automatic addition of 1 N NaOH. The medium was not aerated during the experiment, as this would have led to loss of 2-CVE by vaporization. The initial amount of oxygen present in the headspace of the fermentor was sufficient to keep the culture aerobic during the first 27 h of the experiment.

**Preparation of HOVE from 2-CVE.** Because 2-hydroxyethylvinylether (HOVE) was not commercially available, this compound was synthesized by using purified strain AD25 haloalkane dehalogenase (24). This was done by placing an appropriate amount of enzyme in dialysis tubing in a flask containing 50 mM Tris-sulfate buffer (pH 8.0) and 20 mM 2-CVE at 30°C. At this pH, the spontaneous hydrolysis of 2-CVE was avoided. About 16 mM HOVE was formed routinely, with 4 mM 2-CVE remaining in the solution. The purity of the HOVE was checked by performing a gas

chromatographic analysis. In addition to HOVE, traces of 2-methyl-1,3-dioxolane were formed by a spontaneous reaction of HOVE in water.

**Gas chromatography.** Volatile compounds were analyzed quantitatively by capillary gas chromatography. The equipment and the temperature program used have been described previously (22). Culture samples (4.5 ml) were extracted with 1.5 ml of diethylether containing 0.05 mM 1-bromohexane as the internal standard. The ether layer was analyzed by split injection of 1- $\mu$ l samples. The accuracy (standard deviation) of this method was better than 8%.

The acetaldehyde and VAA in culture samples were analyzed after the cells were removed by centrifugation of 2-ml samples in an Eppendorf centrifuge at  $15,000 \times g$  at 4°C for 5 min and each supernatant was mixed with 0.1 volume of ice-cold 2-bromoethanol (1 mM), which was used as the internal standard. Acetaldehyde and VAA concentrations were determined by split injection of 0.5- $\mu$ l portions of water samples into the gas chromatograph, using a WCOT fused silica column (type FFAP-CB; 25 m by 33 mm; Chrompack, Middleburg, The Netherlands). The following temperature program was used: 3 min at 80°C, followed by increases at a rate of 10°C/min to 200°C. The relative concentrations of VAA (peak area of VAA/peak area of the internal standard) were determined.

Chloroethers were extracted from slurries as follows. A 2.5-ml portion of slurry was mixed with 2.5 ml of acetone and incubated for 15 min in an ultrasonic bath, and then 5 ml of water was added. After incubation for 15 min in the ultrasonic bath, 5 ml of pentane containing 0.1 mM dodecane as an internal standard was added, and the mixture was vortexed. A 1- $\mu$ l portion of the pentane layer was injected in the gas chromatograph for analysis.

The equipment and method used for gas chromatography-mass spectrometry have been described previously (22).

**Chemicals.** All of the chemicals used except HOVE and VAA were obtained from commercial sources (Janssen Chimica or Aldrich) and were at least 95% pure, as determined by gas chromatography (22, 24).

## RESULTS

**Degradation of chloroethers in slurries.** The degradation of several chlorinated ethers was studied in slurries which were prepared with activated sludge or with brackish water sediment. The chloroethers added were 2-CVE, bis(2-chloroethyl)ether, 2-chloroethylethylether, 2-chloroethylpropylether, and 2-chloroethylmethylether. After 12.5 days of incubation, the concentrations of 2-chloroethylpropylether and 2-chloroethylmethylether in activated sludge were somewhat lower than the concentrations in the controls (Table 1). In the incubation mixtures inoculated with brackish water sediment, 2-CVE and bis(2-chloroethyl)ether were degraded (Table 1); this was not observed with the other chloroethers tested.

We attempted to obtain enrichment cultures from these primary incubation mixtures by transferring samples to fresh medium. Degradation of bis(2-chloroethyl)ether, however, did not occur in enrichment cultures as determined by gas chromatographic analyses. Growth on 2-CVE (1 mM) was detected after several subcultivations. Two different pure cultures were isolated and designated strains AD25 and AD27. Both of these bacterial cultures were identified as *A. aquaticus* strains, as described previously (24).

**Characterization of 2-CVE-utilizing cultures.** Growth of strains AD25 and AD27 in liquid media was observed on

TABLE 1. Microbial degradation of halogenated ethers

Haloether	Concn (mM)							
	Activated sludge		Autoclaved activated sludge		Brackish water sediment		Autoclaved brackish water sediment	
	Zero time	12.5 Days	Zero time	12.5 Days	Zero time	14 Days	Zero time	10 Days
2-CVE	0.57	0.17	0.51	0.24	0.37	0.01	0.46	0.13
Bis(2-chloroethyl)ether	0.36	0.27	0.45	0.32	0.35	0.00	0.51	0.39
2-Chloroethylethylether	0.40	0.31	0.49	0.33	0.48	0.43	0.40	0.39
2-Chloroethylpropylether	0.37	0.27	0.38	0.38	0.38	0.35	0.36	0.34
2-Chloroethylmethylether	0.45	0.38	0.51	0.51	0.48	0.45	0.42	0.39

methoxyethanol, ethoxyethanol, methylacetate, ethylene glycol, glycolic acid, and glyoxylic acid. The strains were also able to grow on 1,2-dichloroethane, 2-chloroethanol, chloroacetic acid, methanol, and ethanol. Strain AD25 grew weakly on acetaldehyde. No growth was observed on diethylene glycol, polyethylene glycol, diglycolic acid, and ethoxyacetic acid. Growth of both *A. aquaticus* strains was also observed with 2-chloroethylmethylether but not with any of the other halogenated ethers tested, including bis(2-chloroethyl)ether, chloroethylpropylether, chloroethylethylether, and epichlorohydrin.

The patterns of growth on 4 mM 2-CVE of strains AD25 and AD27 were very similar (Fig. 1), but the yield of strain AD27 was higher than the yield of strain AD25. Growth of the two *A. aquaticus* strains on 2-CVE was nonexponential (Fig. 1). This was not due to a shortage of medium or to poor

growth conditions, because the strains were able to grow exponentially under identical conditions on various substrates, such as glucose, 1,2-dichloroethane, or 2-chloroethanol (24). Within 6 days, 2-CVE was almost completely degraded in both cultures, and equimolar concentrations of HCl were formed (Fig. 1). The pH of both cultures decreased to 6.5. During this period, 2.5 to 3.0 mM acetaldehyde accumulated in the cultures, indicating that the ether bond was cleaved. The strains continued to grow at the expense of acetaldehyde until day 13. 2-Chloroethanol was not found in either culture.

When precultures of strains AD25 and AD27 were grown on 2-CVE and used as inocula for batch cultures growing on 2-CVE, low amounts of HOVE (up to 60  $\mu$ M) accumulated in the new cultures during the first days of growth on 2-CVE. HOVE was utilized again within the first 100 h.

**Dehalogenation in crude extracts.** The dehalogenating activities of strains AD25 and AD27 for several haloethers were investigated with crude cell extracts. Since the yield on 2-CVE was low, cells were grown on 2 mM glucose, and 24 h before harvest 1.5 mM 2-CVE was added to induce 2-CVE metabolism.

Halide release was observed in incubation mixtures of crude extracts and 2-CVE, 2-chloroethylmethylether, 2-bromoethylethylether, or epichlorohydrin (Table 2). Racemic epichlorohydrin was quantitatively dechlorinated, indicating that the enzyme was not stereoselective for this compound. The products of dechlorination appeared to be the corresponding alcohols, indicating that hydrolytic cleavage occurred. In strain AD25 extracts, the specific activities of the dehalogenase with halogenated ethers were about four times higher than the specific activities observed in strain AD27 extracts. No activity was found with bis(2-chloroethyl)ether, 2-chloroethylethylether, or 2-chloroethylpropylether.

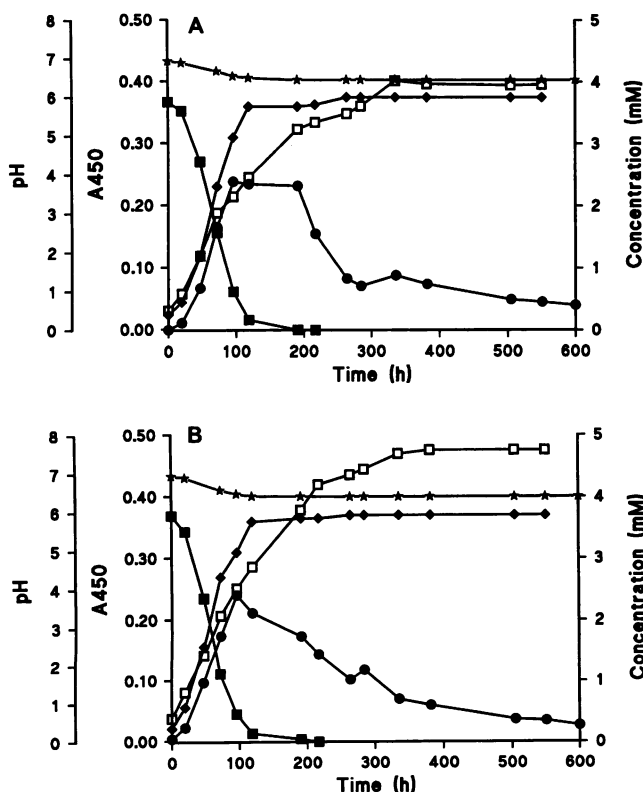


FIG. 1. Growth of strain AD25 (A) and AD27 (B) on 4 mM 2-CVE. Symbols: ■, 2-CVE concentration; ◆, chloride concentration; ●, acetaldehyde concentration; □,  $A_{450}$ ; \*, pH.

TABLE 2. Dehalogenase activities in crude extracts of strains AD25 and AD27

Substrate	Activity (mU/mg of protein) in:		Product
	Strain AD25 <sup>a</sup>	Strain AD27 <sup>a</sup>	
1,2-Dichloroethane	4,710	865	2-Chloroethanol
2-CVE	655	82	2-Hydroxyethylvinylether
2-Chloroethylmethylether	323	46	2-Hydroxyethylmethylether
2-Bromoethylethylether	697	ND <sup>b</sup>	2-Hydroxyethylethylether
Epichlorohydrin	264	78	Glycidol

<sup>a</sup> Cells were grown on 2 mM glucose and were induced by adding 1.5 mM 2-CVE 24 h before the crude extracts were prepared.

<sup>b</sup> ND, not determined.

TABLE 3. Alcohol dehydrogenase activities in crude extracts of strains AD25 and AD27 and *X. autotrophicus* GJ10

Substrate	Sp act (mU/mg of protein) in:			
	Strain AD25 (glucose) <sup>a</sup>	Strain AD25 (2-CVE) <sup>b</sup>	Strain AD27 <sup>a</sup>	Strain GJ10 <sup>c</sup>
Methanol	529	196	332	504
2-Chloroethanol	402	242	312	830
Methoxyethanol	347	ND <sup>d</sup>	222	313
Ethoxyethanol	327	ND	241	462
Diethylene glycol	373	ND	200	163
Ethylene glycol	118	ND	121	0
Hydroxyethylvinylether	405	196	204	694
Ethoxyacetic acid	2	ND	0	0

<sup>a</sup> Cells were grown on 2 mM glucose and were induced by adding 1.5 mM 2-CVE 24 h before the crude extracts were prepared.

<sup>b</sup> Cells were grown on 5 mM 2-CVE.

<sup>c</sup> Specific activity of purified alcohol dehydrogenase from *X. autotrophicus* GJ10 (12).

<sup>d</sup> ND, not determined.

The purified dehalogenase of strain AD25 (24) converted 2-CVE to HOVE, which was identified by gas chromatography-mass spectrometry. Chemical ionization mass spectrometry revealed a molecular ion peak at  $m/z$  88, which corresponds to a molecular formula of  $C_4H_8O_2$ . The mass spectrum contained fragment ions at  $m/z$  45 (relative intensity 100%,  $C_2H_5O$ ),  $m/z$  44 (99%,  $C_2H_4O$ ),  $m/z$  43 (95%,  $C_2H_3O$ ), and  $m/z$  57 (9.5%,  $C_3H_5O$ ).

Minor quantities of 2-methyl-1,3-dioxolane (2-MDO) were also produced in these assays. The gas chromatography-mass spectrometry spectrum was identical to that of commercially obtained 2-MDO. The formation of 2-MDO was a result of the spontaneous conversion of HOVE in water. This reaction also proceeded in diethylether and has been found to occur in tetrachloromethane as well (21). 2-MDO was not converted by strains AD25 and AD27 during or after growth on 2-CVE. A solution containing 0.4 mM HOVE decreased 8 to 10% in concentration during 24 h of incubation in 50 mM Tris-sulfate buffer (pH 7.5) at 30°C, indicating that the half-life was  $179 \pm 21$  h.

The dehalogenases of strains AD25 and AD27 and of *X. autotrophicus* GJ10 are identical and also dechlorinate 1,2-dichloroethane with the formation of 2-chloroethanol and HCl (24). With purified enzyme, a  $V_{max}$  of 2.6 mU/mg of protein and a  $K_m$  of 10 mM were found with 2-CVE as the substrate.

**Acid-catalyzed hydrolysis of 2-CVE and HOVE.** The stability of 2-CVE in buffer was checked since it has been reported that hydrolysis to acetaldehyde and 2-chloroethanol may occur (3). The half-lives of 2-CVE in 50 mM Tris-sulfate buffer at 30°C were 37 days at pH 7.5, 19 days at pH 7, and 7.5 days at pH 6.5. Consequently, during growth on 4 mM 2-CVE a significant amount of 2-CVE (but less than 1 mM) is hydrolyzed chemically to acetaldehyde and 2-chloroethanol.

**Alcohol dehydrogenase activities.** Crude extracts of the two strains contained phenazine methosulfate-dependent alcohol dehydrogenase activity that oxidized methanol, 2-chloroethanol (24), and several alcohol ethers, including HOVE (Table 3). The product of HOVE presumably was vinyloxyacetaldehyde. No activity was found with ethoxyacetic acid.

The purified quinoprotein alcohol dehydrogenase of *X. autotrophicus* GJ10, which is capable of oxidizing methanol and 2-chloroethanol (10), also converted HOVE, methoxyethanol, ethoxyethanol, and diethylene glycol (Table 3).

TABLE 4. Acetaldehyde dehydrogenase activities in crude extracts of strains AD25 and AD27

Substrate	Sp act (mU/mg of protein) in:	
	Strain AD25 <sup>a</sup>	Strain AD27 <sup>a</sup>
Chloroacetaldehyde	2,350	492
Acetaldehyde	1,780	454
Methoxyacetaldehyde	1,700	509

<sup>a</sup> Cells were grown on 2 mM glucose and were induced by adding 1.5 mM 2-CVE 24 h before the crude extracts were prepared. No activity was found with ethoxyacetic acid, methoxyacetic acid, or diglycolic acid.

Thus, this type of alcohol dehydrogenase may be involved in the oxidation of the alcohol ethers in the facultative methylotrophs which we studied.

**Aldehyde dehydrogenase activities.** Both *A. aquaticus* AD25 and *A. aquaticus* AD27 produced an aldehyde dehydrogenase that possessed activity for acetaldehyde, chloroacetaldehyde, and methoxyacetaldehyde (Table 4). The activity in strain AD25 cells growing on glucose is low (24). The enzyme was induced to very high levels by the addition of 2-CVE to such cultures (Table 4).

Vinyloxyacetaldehyde was not tested as a substrate since it was not commercially available. The related compound methoxyacetaldehyde was oxidized in crude extracts at rates that were 95 and 112% of the velocities found with acetaldehyde in strains AD25 and AD27, respectively.

**Formation of VAA by cell suspensions of strain AD25.** To investigate whether strain AD25 converted 2-CVE to VAA, a 600-ml cell suspension of strain AD25 (0.4 mg [dry weight] per ml) was prepared from cells grown on 2 mM glucose in batch cultures and incubated in a fermentor with 10 mM 2-CVE (see Materials and Methods). After 27 h, approximately 8.5 mM 2-CVE was degraded, and 8.5 mM HCl was formed. No HOVE or 2-MDO was detected. Instead, large quantities of a non-diethylether-extractable compound accumulated in this period. Chemical ionization mass spectrometry of this compound revealed a molecular ion peak at  $m/z$  102, which corresponds to a molecular formula of  $C_4H_6O_3$ . The mass spectrum contained fragment ions at  $m/z$  43 (relative intensity, 100%,  $C_2H_3O$ ),  $m/z$  87 (58%,  $C_3H_3O_3$ ),  $m/z$  59 (32%,  $C_2H_3O_2$ ),  $m/z$  58 (23%,  $C_2H_2O_2$ ),  $m/z$  44 (15%,  $C_2H_4O_2$ ), and  $m/z$  42 (14%,  $C_2H_2O$ ). The molecular formula  $C_4H_6O_3$  and the fragment pattern are consistent with the formula and fragment pattern of VAA.

In addition to VAA, acetaldehyde was also detected in this culture. After 12 h, about 1.9 mM acetaldehyde had accumulated. After 27 h, the concentration of acetaldehyde was 1.8 mM.

**Degradation of VAA.** To investigate whether strain AD25 was able to convert VAA, a fermentor medium that contained a high concentration of VAA was separated from the cells by centrifugation at  $10,000 \times g$  for 15 min and filter sterilized. New cells of strain AD25 were grown on 2 mM glucose, and this was followed by incubation for 30 h with 1 mM 2-CVE. Then the cells were harvested by centrifugation and resuspended in the supernatant that contained VAA. The concentration of VAA in this incubation mixture decreased about 20% over a period of 5 days (Fig. 2), indicating that strain AD25 had no or only very low activity for the degradation of VAA.

**Ether cleavage.** Crude extracts prepared from cells of strains AD25 and AD27 grown on a mixture of glucose and 2-CVE were screened for ether-cleaving activities. The

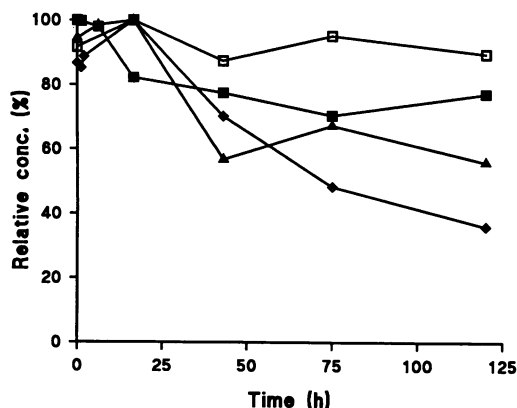


FIG. 2. Degradation of VAA. Symbols: ■, incubation with strain AD25 (0.35 mg [dry weight] per ml), pH 7.1; □, incubation with no cells, pH 7.8; ▲, incubation with no cells, pH 7.1; ◆, incubation with no cells, pH 6.6.

ethers used in these assays were diglycolic acid, ethoxyacetic acid, and methoxyacetic acid. Diglycolic acid dehydrogenase activity linked with 1,2-dichlorophenolindophenol and phenazine methosulfate (13) was not found in the crude extracts of the two strains. Moreover, oxidase activity (assayed with NADH) (13) and oxygenase activity (assayed with NADH or NADPH) (13) were also not observed in the crude extracts of the two strains.

**Alcohol dehydrogenase-negative mutant AD25M3.** To test whether the ether bond of 2-CVE or HOVE can be cleaved by strain AD25, an alcohol dehydrogenase-negative mutant of strain AD25 was isolated. Mutants were selected on mineral salts medium agar supplemented with 30 mg of yeast extract per liter, 5 mM glucose, and 0.5 mM 2-bromoethanol. The latter compound is a suicide substrate, presumably because of conversion to bromoacetaldehyde (9). A 2-bromoethanol-resistant mutant designated strain AD25M3 was chosen for further study. Replica plating showed that in contrast to strain AD25, strain AD25M3 did not grow on methanol, methoxyethanol, ethoxyethanol, 2-chloroethanol, or 1,2-dichloroethane. Strain AD25M3 was able to grow on chloroacetic acid and grew slowly on ethanol. Revertants of AD25M3 that grew on methanol frequently developed within a few weeks. Extracts prepared from strain AD25M3 grown on glucose did not contain PMS-linked alcohol dehydrogenase. In contrast, extracts of glucose-grown strain AD25 cells contained 1.97 U of dehydrogenase per mg of protein (24).

A batch culture of strain AD25M3 grown on 2 mM glucose was supplemented with 1.6 mM 2-CVE. During degradation of 2-CVE, gradual accumulation of HOVE up to a concentration of 1.45 mM was observed, and equimolar concentrations of HCl were produced (Fig. 3). In addition, 32  $\mu$ M 2-MDO, 0.27 mM acetaldehyde, and some VAA were produced. 2-Chloroethanol was not found.

Our results confirmed that 2-CVE is converted by strain AD25 to HOVE. The finding that after 27.5 h 85% of the expected amount of HOVE was still present in the culture medium indicated that strain AD25M3 was not able to convert this compound. Low amounts of acetaldehyde were produced, indicating that there was some hydrolysis of HOVE to acetaldehyde and ethylene glycol. The latter compound, however, was not found. Furthermore, low concentrations of VAA were produced by strain AD25M3,

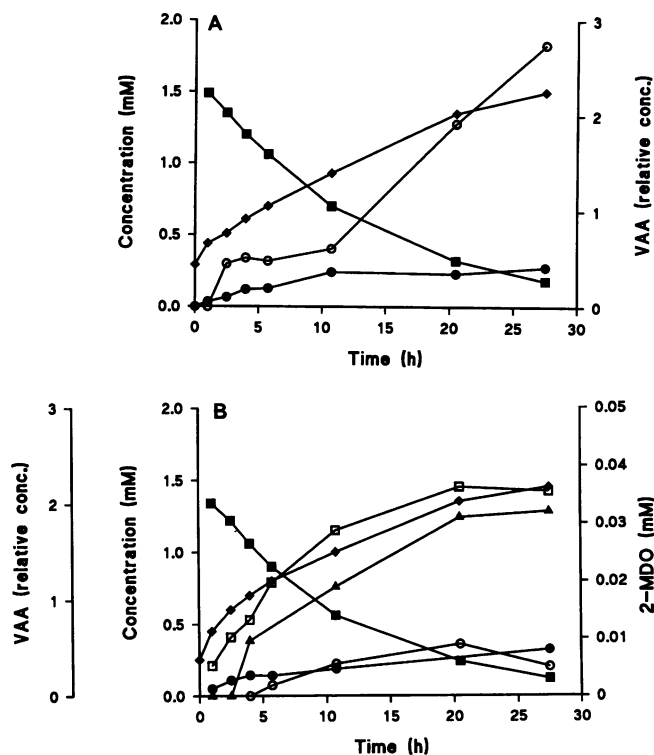


FIG. 3. Degradation of 1.6 mM 2-CVE in cultures of strains AD25 (A) and AD25M3 (B) which were pregrown on 2 mM glucose. Symbols: ■, 2-CVE concentration; □, HOVE concentration; ●, acetaldehyde concentration; ◆, chloride concentration; ▲, 2-MDO concentration; ○, VAA concentration.

indicating that a small amount of alcohol dehydrogenase activity was present in the mutant, which was confirmed in a parallel experiment in which strain AD25M3 slowly degraded 0.4 mM 2-chloroethanol over a 27-h period. In control experiments, strain AD25 converted about 1.3 mM 2-CVE to an equimolar amount of HCl, 0.32 mM acetaldehyde, and some VAA (Fig. 3). HOVE was not found in these cultures.

## DISCUSSION

Biodegradation and enrichment tests performed with five different chloroethers in prolonged incubations with brackish water sediment or with activated sludge revealed that 2-CVE could be a growth substrate. Of the other chloroethers tested, only bis(2-chloroethyl)ether was converted completely in brackish water sediment, but this compound was not used as a growth substrate during further enrichment. Whereas most of the chloroethers tested seemed to be resistant to biodegradation, the alcohol ethers ethoxyethanol and diethyleneglycol are good growth substrates for a variety of cultures (2, 14). Thus, the key steps are the initial dehalogenation reactions.

Two different *A. aquaticus* strains, AD25 and AD27, are able to use 2-CVE as a sole carbon and energy source. Dehalogenation was catalyzed by a haloalkane dehalogenase that is also produced by several 1,2-dichloroethane-utilizing bacterial cultures (24). This enzyme converts halogenated aliphatic compounds by a nucleophilic substitution reaction to alcohols and inorganic halide (5, 12, 16). The *dhL4* genes

are identical in all 1,2-dichloroethane-utilizing strains that have been investigated (11) and are located on similar 8.3-kb *EcoRI* restriction fragments (11, 24). The enzyme has a broad substrate specificity. As of now, some 30 different halogenated aliphatic compounds, including several haloethers, have found to be substrates of the enzyme. A different haloalkane dehalogenase that acts also on chloroethers has been purified from gram-positive strain GJ70 (9). This enzyme also converts 2-CVE and bis(2-chloroethyl)ether.

HOVE produced from 2-CVE by strains AD25 and AD27 was oxidized to ethoxyacetaldehyde, a reaction catalyzed by a quinoprotein alcohol dehydrogenase. Purified quinoprotein alcohol dehydrogenase of *X. autotrophicus* GJ10 oxidized methanol, 2-chloroethanol (10), HOVE, and other alcohol ethers. Oxidation of alcohol ethers has also been observed with purified quinoprotein methanol dehydrogenases of methanotrophs (1). Moreover, polyethylene glycols are converted predominantly by alcohol dehydrogenases to their corresponding aldehydes (2, 14, 15).

Another route for the conversion of alcohol ethers is cleavage of the ether bridge. It has been proposed that HOVE is an intermediate that undergoes ether cleavage during the degradation of diethylene glycol by an *Acinetobacter* sp. (19). A membrane-bound oxygen-sensitive diethylene glycol lyase produced acetaldehyde and ethylene glycol from polyethylene glycol. However, in strain AD25 and AD27 cultures grown on 2-CVE ethylene glycol was not found. The alcohol dehydrogenase-negative mutant AD25M3 grown on glucose did not degrade HOVE produced from 2-CVE (Fig. 3), indicating that this intermediate did not undergo ether cleavage. Instead, small amounts of 2-MDO, a cyclic compound, were formed from HOVE in these cultures. This was due to a spontaneous intramolecular addition reaction of HOVE, which was also observed in the absence of cellular protein.

Formation of VAA from 2-CVE was observed with strain AD25 cells grown on glucose and with washed strain AD25 and AD27 cells grown on 2-CVE (data not shown). An aldehyde dehydrogenase of strains AD25 and AD27 capable of oxidizing methoxyacetaldehyde is thought to be responsible for the production of VAA from vinyloxyacetaldehyde. Thus, the enzymes of both *A. aquaticus* strains involved in the degradation of 2-CVE are haloalkane dehalogenase, alcohol dehydrogenase, and aldehyde dehydrogenase (Fig. 4).

Degradation of different polyethylene glycols by a variety of pure bacterial cultures has been shown to proceed similarly. The alcohol ether is converted by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding acid (2, 6, 14, 15), and then ether cleavage takes place (2, 13, 14). However, enzymatic ether cleavage was not observed with strain AD25 and AD27 cells. Consequently, growth seemed to occur by utilization of the products formed from VAA by chemical decomposition, most probably acetaldehyde and glycolic acid (Fig. 4). This does not explain why VAA did not accumulate during growth on 2-CVE, and the possibility that an unidentified ether-metabolizing activity is present cannot be excluded.

As described previously, strains AD25 and AD27 were also able to grow on 1,2-dichloroethane (24). The conversion of 2-CVE to VAA and the conversion of 1,2-dichloroethane to chloroacetic acid appear to be carried out by similar enzymes.

Strains AD25 and AD27 also produced chloroacetic acid during growth on 2-CVE as a result of the formation of

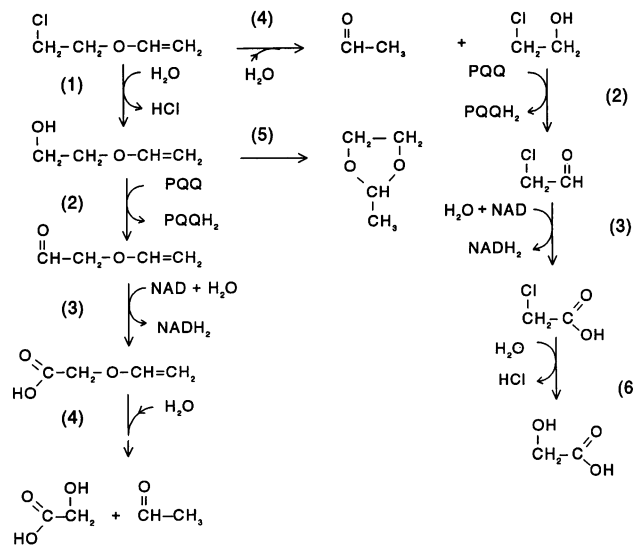


FIG. 4. Proposed route of the degradation of 2-CVE by strains AD25 and AD27. 1, haloalkane dehalogenase; 2, alcohol dehydrogenase; 3, aldehyde dehydrogenase; 4, chemical hydrolysis; 5, intramolecular addition; 6, chloroacetic acid dehalogenase.

2-chloroethanol by chemical hydrolysis of 2-CVE. This may explain why both strains also have chloroacetic acid dehalogenase activity and as a result possess the whole route for degradation of 1,2-dichloroethane to glycolate.

At pH 7, the half-life of 2-CVE is 19 days, indicating that under these conditions the second route for the metabolism of 2-CVE probably is not very important. However, a decrease in the pH of the medium decreased the half-life of 2-CVE, indicating that under these conditions metabolism of 2-CVE via 2-chloroethanol is important. Thus, two different routes for the degradation of 2-CVE have been recognized, and both may lead to the products acetaldehyde and glycolic acid (Fig. 4), which can be used as growth substrates.

The specific activity of the haloalkane dehalogenase for 2-CVE was much lower than the specific activity for 1,2-dichloroethane ( $k_{cat}/K_m$ , 152 and 4,853  $M^{-1} s^{-1}$ , respectively). The overproduction of the enzyme by strains AD25 and AD27 may be an adaptation to compensate for this low selectivity for 2-CVE. We have recently found that strain AD25, because of its high haloalkane dehalogenase content, has a Monod constant for 1,2-dichloroethane which is 10-fold lower than the Monod constant of 1,2-dichloroethane utilizers that produce lower levels of the haloalkane dehalogenase, such as *X. autotrophicus* GJ10 (21a). Thus, bacterial enrichment on a compound that is a poor substrate for the haloalkane dehalogenase led to selection of strains that have an improved affinity for 1,2-dichloroethane.

Chlorinated ethers, diethers, and triethers may be rather resistant to dehalogenation in contaminated soils and sediments (4). In the absence of suitable dehalogenating activities, enzymatic ether cleavage may be the first step. When these reactions are carried out by aspecific oxygenases (1) in nature, halogenated alcohols may be produced from halogenated ethers. Chlorinated alcohols have been shown to be growth substrates for a variety of microorganisms (12, 22, 24, 25).

In this study we found that some cultures of *A. aquaticus* are able to grow on halogenated ethers. Since these organisms are also able to degrade other halogenated aliphatic



compounds (24), bacteria of the genus *Ancylobacter* may have potential for application in waste treatment and may play an important role in the removal of halogenated aliphatic compounds from the natural environment.

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